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(54) Title: ISOPROSTANE-PROTEIN CONJUGATES

(57) Abstract

The present invention provides novel isoprostane-protein conjugates. By virtue of their antigenicity and their ability to act as tracer molecules in enzyme immunoassay procedures, they represent important new diagnostic agents that permit the measurement of isoprostanes in biological samples.

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ISOPROSTANE-PROTEIN CONJUGATES

FIELD OF THE INVENTION

The present invention is directed to novel isoprostane-protein conjugates which enable the measurement of isoprostanes by enzyme-immunoassay techniques and are useful in producing specific antiserum and antibodies for isoprostanes.

BACKGROUND OF THE INVENTION

Isoprostanes are structural derivatives of isoprostanoic acid and are naturally occurring biomolecules which have been reported in biochemical literature for many years. See Svanborg et al, Biomed. Mass Spectrom., 10 (9), pp. 495-498 (1983). J. D. Morrow et al, Proc. Natl. Acad. Sci., Vol. 87, pp. 9383-9387 (1990), has described isoprostanes as a class of eicosanoids produced non-enzymatically by the random oxidation of cellular lipids.

It has recently been discovered that isoprostanes are not cyclooxygenase metabolites and therefore are unrelated to the prostaglandins and thromboxanes of enzymatic origin. Rather, the isoprostanes are structurally unique eicosanoids whose formation coincides with nonspecific oxidative tissue damage. Thus, they present distinctly novel and different medical and biochemical implications. Isoprostane measurement is important to allow improved diagnosis of all cases of oxidative stress and oxidative tissue damage, including ischemic tissue re-perfusion injury, oxidatant stress from environmental sources such as ozone pollution or intoxication, and oxidative lung injury in the respiratory distress syndromes of prematurity and

adult acute pulmonary trauma. For example, the measurement of isoprostane $F_2\alpha$ in victims of drowning, asphyxiation or fire would allow improved assessment of the extent of damage to vital internal organs. The measurement of isoprostane $F_2\alpha$ in victims of myocardial infarction would give an improved measurement of the extent of the damage to the heart tissue and help predict prognosis and optimal treatment. The measurement of isoprostane $F_2\alpha$ in victims of stroke, closed head injury and cold water immersion would provide a better measurement of the degree of brain injury and the likelihood of survival. Isoprostane measurements have been made in the past, notably by gas chromatography/mass spectrometry. See K. Svanborg, M. Bygdeman, P. Enroth, Biomed. Mass Spectrom., 10 (9) pp. 495-498 (1983). However, this technique is cumbersome, tedious and expensive. It is unsuited to the large volume and limited cost required for a viable medical diagnostic test.

The art of chemically linking small molecules such as steroids, thyroid hormones and peptides to proteins is well known. See Handbook of Experimental Pharmacology, C. Patrono and B. Peskar, eds. Springer-Verlag, New York, Vol. 82, pp. 23-61 and pp. 143-175. For example, the steroid hormone progesterone has been chemically linked to bovine serum albumin, rendering a conjugate capable of eliciting antiprogesterone antibodies when injected into rabbits. However, until the present invention, there have been no reports of the production or utilization of isoprostane-protein conjugates.

SUMMARY OF THE INVENTION

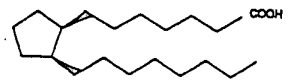
The present invention relates to novel protein conjugates wherein isoprostanes are linked covalently to enzymes and antigenic peptides, forming conjugates useful

for the production of specific antiserum and antibodies. The protein conjugates of the present invention are heteromultimers consisting of two parts. The first part is the isoprostane molecule. The second part is a protein molecule, which may be acetyl cholinesterase, bovine serum albumin, keyhole limpet hemocyanin, porcine thyroglobulin, horseradish peroxidase, alkaline phosphatase, β-galactosidase, glucose oxidase, urease, glucose-6-phosphate dehydrogenase and penicillinase.

The novel protein conjugates of the present invention would make the precise, accurate and inexpensive measurement of isoprostanes possible. Furthermore, isoprostane-protein conjugates may be useful in the studies of isoprostane binding proteins and receptors which may play an important role in the above physiological and pathological conditions.

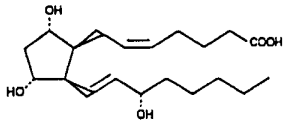
DETAILED DESCRIPTION OF THE INVENTION

The isoprostanes of the present invention are derivatives of isoprostanoic acid, which is shown below as formula I.



I

Especially preferred isoprostanes used in the present invention are 8-isoprostane $F_2\alpha$, 8-isoprostane E_2, 8-isothromboxane B_2 and 9β,11β-8-isoprostane F_2 which are shown below as formulas II - V, respectively.



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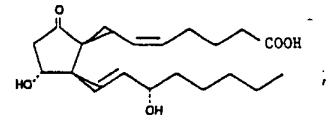

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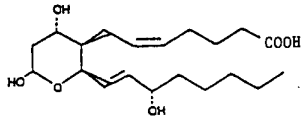

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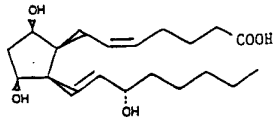
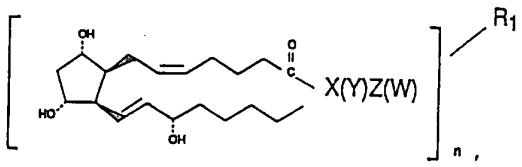


V

The preferred isoprostane-protein conjugates of the present invention are of the following formulas VI-IX:



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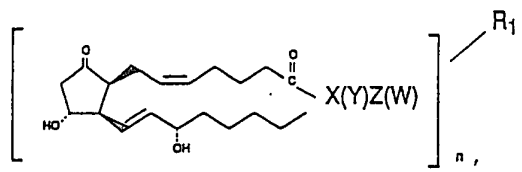

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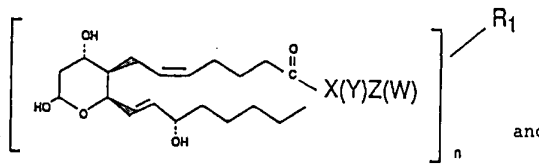

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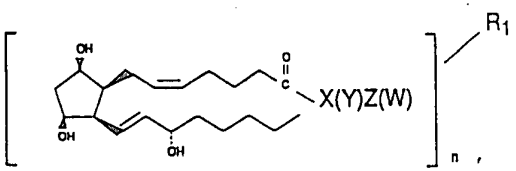
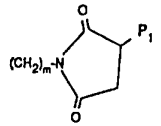
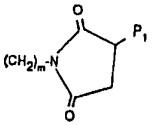


IX

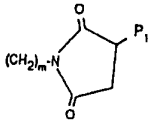
wherein X is O, NH, N, CH_2, S or NHCO; Y is a single or double covalent bond, a straight chain or branched alkyl group having from 1 to 12 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, a phenyl group, $CO(CH_2)CO$, a succinamide group of the formula



, with m being an integer of 0-10 and P_1 is S, NH or O, bisdiazobenzidine, NH, N, S, CO or O; Z is a single or double covalent bond, a straight chain or branched alkyl group having from 1 to 12 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, a phenyl group, $CO(CH_2)CO$, a succinamide group of the formula



, with m being an integer of 0-10 and P_1 is S, NH or O, bisdiazobenzidine, NH, N, S, CO or O; W is a single or double covalent bond, a straight chain or branched alkyl group having from 1 to 12 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, a phenyl group, $CO(CH_2)CO$, a succinamide group of the formula



, with m being an integer of 0-10 and P_1 is S, NH or O, or bisdiazobenzidine; R^1 is acetyl cholinesterase, horseradish peroxidase, alkaline

phosphatase, β-galactosidase, glucose oxidase, urease, glucose-6-dehydrogenase, penicillinase, serum albumins, thyroglobulins or keyhole limpet hemocyanin, and n is an integer of 1-100.

The peptide conjugates of isoprostanes of the present invention represent novel molecules that have many important diagnostic uses. For example, the peptide conjugate of isoprostane $F_2\alpha$ with bovine serum albumin, thyroglobulin or keyhole limpet hemocyanin would be antigenic in rabbits and would be useful in preparing specific rabbit antiserum against isoprostane $F_2\alpha$. Thus, by using this novel substance and following immunologic techniques well known to those skilled in the art, antisera specific for 8-isoprostane $F_2\alpha$ has been produced. Such antigenic conjugates would also elicit an antibody response in mice, allowing the production of specific monoclonal antibodies to the isoprostanes through hybridoma techniques known to those skilled in the art. Further, the enzyme conjugates of isoprostane $F_2\alpha$ with electric eel acetyl cholinesterase, horseradish peroxidase, or alkaline phosphatase provide novel enzymatic tracers for use in immunodiagnostic measurement. The combination of the specific rabbit antiserum or mouse monoclonal antibody against isoprostane $F_2\alpha$ with the enzyme conjugates of isoprostane $F_2\alpha$ with the use of enzyme immunoassay techniques familiar to those skilled in the art permit the precise immunodiagnostic measurement of isoprostane $F_2\alpha$ in medical and biological samples.

The preparation of the isoprostane-protein conjugates is illustrated by the following examples.

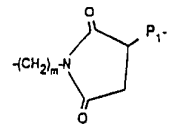
Example 1

The conjugate of formula III wherein X is NH, Y, Z and W are a single covalent bond; R is acetyl cholinesterase and n = 1 - 6 is prepared as follows:

To 10 μL of 10 mM isoprostane $F_2\alpha$ in dimethylformamide was added 10 μL of coupling reagent (1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 50 mM N-hydroxy-sulfosuccimide in 0.1 M potassium phosphate buffer pH 7.4). 100 μg of acetylcholinesterase in 100 μL 0.1 M potassium phosphate buffer pH 7.4 was added. The mixture was incubated at room temperature overnight. After incubation, the free small molecules were removed by gel chromatography with a Sephadex G-10 column. The fractions are monitored by detection of the UV absorbance at 280 nm, and those corresponding to the G4 form of acetylcholine-sterase are combined, diluted 1:1000, and used as an enzymatic tracer for the analysis of isoprostane $F_2\alpha$ in the range of 10 - 500 pg/ml.

Example 2

The conjugate of formula III wherein X is NHCO, Y is cyclohexyl, Z is a succinamide of the formula



where P_1=S and m=1, W is a single covalent bond and R is bovine serum albumin and n = 10 - 100 is prepared as follows:

To 10 μL of 10 mM isoprostane $F_2\alpha$ in acetone was added 10 μL of 10 mM isobutylchloroformate and 10 μL of 10 mM of Diisopropylethylamine. The reaction mixture was incubated at 0°C for 30 minutes and 70 μL of 10 mM ethylene diamine was added at -20°C. The reaction mixture was allowed to

warm to room temperature in two hours. The solvent was removed after evaporation under reduced pressure. The crude product was dissolved in 20 μL of dimethylformamide, and a 10 μL of 10 mM succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate was added. The mixture was incubated at room temperature for one hour and 100 μg of bovine serum albumin in 100 μL of 0.1M potassium phosphate buffer (pH 8.0). The mixture was left at 4°C overnight. Free small molecules were removed by repeated dialysis against phosphate buffered saline. The resulting solution was concentrated to 1 ml of total volume and emulsified with Freunds complete adjuvant in a ratio of 1:1 to give an immunogen suitable of eliciting specific antibodies to isoprostane $F_2\alpha$ when injected into rabbits.

In the isoprostane-protein conjugates of the present invention, it is desirable for the proteins to be linked to the isoprostanes through an α-side chain of one of the amino acids making up the proteins. That is, where the amino acid is lysine in the protein sequence, the bond is through $(CH_2)_4$; where the amino acid is aspartate, serine or cysteine, the bond is through CH_2; where the amino acid is glutamate, the bond is through $(CH_2)_2$; and where the amino acid is tyrosine, the bond is through the phenyl group.

In practice, the novel compounds of the present invention are used as follows: A microtiter well is coated with antibody that is specific to isoprostanes before starting an assay. Then 50 μl of sample taken from a living specimen is added to the wells. If the living specimen is injured, the sample will contain an amount of isoprostanes proportional to the injury. This is followed by the addition of 50 μl of the isoprostane-protein conjugate of the current invention to the wells. The

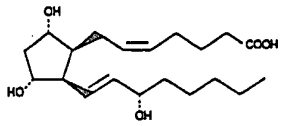
mixture is allowed to incubate wherein an equilibrium develops as both the isoprostane-protein conjugate of the invention and the isoprostanes of the sample (assuming the living specimen from which the sample was taken contains isoprostanes) compete to bind to a limited number of antibody binding sites. Once the equilibrium has been established, the excess isoprostane-protein conjugate and sample are washed away with buffer and only those that have bound to the well remain. Relatively large quantities of isoprostane-protein conjugate will be bound to wells that contain samples having low concentrations of isoprostanes. Conversely, very small amounts of isoprostane-protein conjugates will be bound to wells that contained samples having high concentrations of isoprostanes (indicating injury). The amount of bound isoprostane-protein conjugate molecules can be measured by methods known in the art. The amount of isoprostane-protein conjugate is inversely proportional to the amount of isoprostanes in the sample.

Although a particular preferred embodiment of the invention has been disclosed in detail for illustrative purposes, it will be recognized that variations or modifications of the disclosed apparatus, including the rearrangement of parts, lie within the scope of the present invention.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An isoprostane-protein conjugate comprising an isoprostane covalently bonded to a protein.

2. The isoprostane-protein conjugate of Claim 1, wherein said isoprotane is selected from the group consisting of



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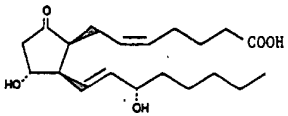

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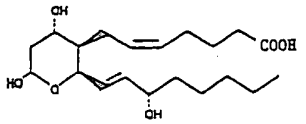

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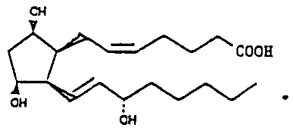
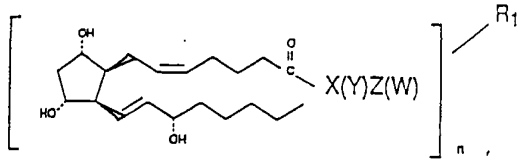


V

3. The isoprostane-protein conjugate of Claim 1, wherein said protein is selected from the group consisting of acetylcholinesterase, horseradish peroxidase, alkaline phosphatase, β-galactosidase, glucose oxidase, urease, glucose-6-dehydrogenase, penicillinase, serum albumins, thyroglobulins and keyhole limpet hematocyanin.

4. The isoprostane-protein conjugate of Claim 1, wherein said isoprostane-protein conjugate is selected from the group consisting of



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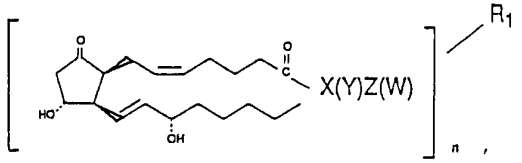

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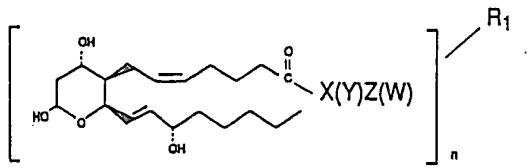

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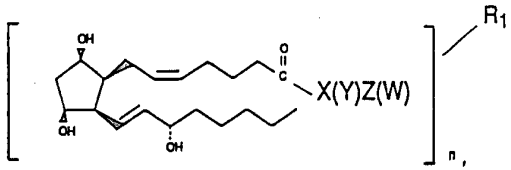
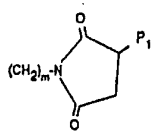
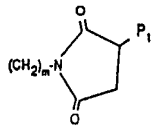


IX

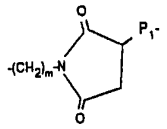
wherein X is O, NH, N, CH_2, S or NHCO; Y is a single or double covalent bond, a straight chain or branched alkyl group having from 1 to 12 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, a phenyl group, $CO(CH_2)CO$, a succinamide group of the formula



, with m being an integer of 1-10 and P_1 is S, NH or O, bisdiazobenzidine, NH, N, S, CO or O; Z is a single or double covalent bond, a straight chain or branched alkyl group having from 1 to 12 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, a phenyl group, $CO(CH_2)CO$, a succinamide group of the formula

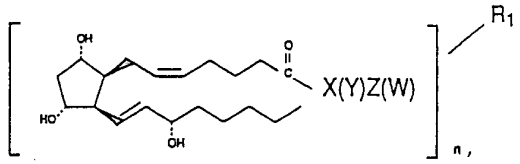


, with m being an integer of 0-10 and P_1 is S, NH or O, bisdiazobenzidine, NH, N, S, CO or O; W is a single or double covalent bond, a straight chain or branched alkyl group having from 1 to 12 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, a phenyl group, $CO(CH_2)CO$, a succinamide group of the formula



, with m being an integer of 0-10 and P_1 is S, NH or O, or bisdiazobenzidine; R^1 is acetyl cholinesterase, horseradish peroxidase, alkaline phosphatase, β-galactosidase, glucose oxidase, urease, glucose-6-dehydrogenase, penicillinase, serum albumins, thyroglobulins and keyhole limpet hematocyanin, and n is an integer of 1-100.

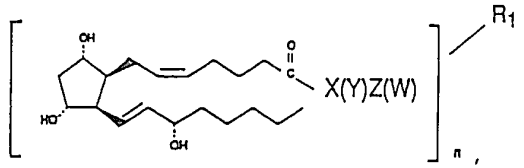
5. The isoprostane-protein conjugate of Claim 4, wherein said isoprostane-protein conjugate is



VI

X is NH; Y, Z and W are one, single covalent bond, R is acetyl cholinesterase, and n is an integer of 1-6.

6. The isoprostane-protein conjugate of Claim 4, wherein said isoprostane-protein conjugate is



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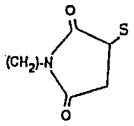
X is NHCO, Y is cyclohexyl, Z is a succinamide of the formula 

, W is a single covalent bond, R is bovine serum albumin, and n is an integer of 10-100.

7. A method of measuring isoprostanes in a biological sample comprising the steps of coating a microtiter well with antibodies specific to isoprostane; adding an amount of isoprostane-protein conjugate and an amount of biological sample to the well; allowing both the isoprostane-protein conjugate and the isoprostanes from the biological sample to compete for binding sites on the antibodies; washing unbound isoprostane-protein conjugates and sample away with a buffer; and determining the quantity

of isoprostanes in the biological sample by measuring the amount of bound isoprostane-protein conjugates.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07630

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/53; C07K 17/00; C08H 1/00
US CL :435/7.9; 530/402, 403

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.9, 188; 530/402, 403, 807, 388.9, 389.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0,166,583 (Taniguchi, et al.) 02 January 1986, see pages 9-13 and Example 3.	1-7
Y	Proc. Natl. Acad. Sci., Vol. 87, issued December 1990, Morrow et al., "A series of Prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism", pages 9383-9387, see abstract and Figure II.	1-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search	Date of mailing of the international search report
28 October 1993	[illegible]3
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer LORA M. GREEN Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07630

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biomedical Mass Spectrometry, Vol. 10, No. 7, issued July 1983, Svanborg et al., "The F and 19-Hydroxy F Prostaglandins and their 8B-Isomers in Human Seminal Plasma: Data on Chromatography and Mass Spectrometry", pages 495-498, see pages 497-498.	1-7

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